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(54) Title: **PROCESS FOR THE PREPARATION OF N-ACETYLNEURAMINIC ACID**

(57) Abstract

A method for the preparation of N-acetyl-D-neuraminic acid (NANA) from N-acetyl-D-glucosamine (NAG) is described.

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PROCESS FOR THE PREPARATION OF N-ACETYLNEURAMINIC ACID

The present invention relates to the preparation of N-acetyl-D-neuraminic acid.

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N-Acetyl-D-neuraminic acid (NANA) is a sialic acid which occurs naturally as a component of complex mucoid substances such as mucolipids and mucoproteins and also as a component of oligosaccharides found for example in milk.

10

NANA is a useful starting material for compounds of interest as therapeutic agents. However, NANA is currently available only in limited quantities and there is a need for processes for its preparation from simple, widely available starting materials. The present invention relates to a method for the preparation of NANA from N-acetyl-D-glucosamine (NAG), and pyruvic acid.

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International Patent Application No. 93/15214 (published 5th August, 1993) discloses a one-pot procedure for the preparation of NANA by treatment of NAG with pyruvic acid and NANA lyase under alkaline conditions.

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The present invention provides in a first aspect a process for the preparation of NANA comprising the steps of :-

25

1. converting NAG to N-acetyl-D-mannosamine (NAM);
2. reacting NAM with pyruvate under enzymatic conditions; and
3. isolating the thus-produced NANA

30

Conversion of NAG to NAM is effected by base-catalysed epimerisation. This reaction may be effected by treating NAG with a base, for example an alkali metal hydroxide such as sodium or potassium hydroxide or a quaternary ammonium ion exchange resin in the hydroxyl form such as Duolite A113 (OH⁻), in an aqueous medium, for example water, at ambient or elevated temperature, for example 20 to 50°C, preferably about 25 to 35°C. Epimerisation may be carried out at different

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concentrations of NAG, but preferably at its saturation concentration. The reaction

is continued until equilibration between NAG and NAM is reached. Reaction times will vary with the temperature and pH of the reaction but for example at about 25°C and about pH11 equilibration is achieved at about 20 - 60, for example about 40, hours, or at about 32°C and >pH11 equilibration is achieved at about 12-20, for example about 16, hours. The ratio of NAM to NAG on equilibration is typically about 1:4 by weight.

When a base such as sodium hydroxide is used in the epimerisation step, the reaction mixture is neutralised using, for example, an acid resin [e.g. Amberlite 200 (H⁺) or IR120 (H⁺)] or a suitable acid (e.g. acetic acid) prior to the second step of the process of this invention.

In one aspect of the invention the NAM/NAG mixture is used directly in the second step of the process.

In an alternative and preferred aspect of the invention the NAM/NAG mixture is enriched in NAM by selective removal of NAG. This enrichment may be carried out in a variety of ways. Thus, the NAM/NAG mixture may be concentrated to a residue by evaporation or spray drying. This residue may then be extracted with an organic solvent such as a lower alcohol, preferably methanol, in an amount just sufficient to dissolve the NAM present. Removal of insoluble NAG gives a solution rich in NAM. Alternatively, the NAM/NAG mixture, preferably having a high starting concentration of NAG or optionally concentrated to achieve a high concentration of NAG, may be treated with a suitable organic solvent such as a lower alcohol, preferably isopropanol, in the ratio of 1 volume of epimerisation mixture to 5 to 10 volumes, preferably about 6 volumes, of isopropanol, and preferably at a temperature of from 15° to 30°C, such as 20° to 25°C. After removal of crystalline NAG the residual solution is rich in NAM. This solution may be further enriched by evaporation of the isopropanol/water azeotrope, preferably with an isopropanol feed to the evaporator, to give a second crop of crystalline NAG. Organic solvent is removed from the NAM-enriched solutions before presenting them to step 2 of the process at the preferred concentration in water. By this means the ratio of NAM to NAG may be increased to about 1.5:1 to 10:1, typically, on large scale, 1.5:1 to 4:1. NAG recovered during the enrichment process may be reused in the epimerisation step, optionally in admixture with fresh NAG.

In the second step of the process the NAM/NAG mixture (preferably enriched in NAM) is incubated with pyruvate (e.g. sodium pyruvate) in the presence of NANA aldolase, for example *Escherichia coli* NANA aldolase (EC4.1.3.3).

- 5 NANA aldolase is a known enzyme [cf D Comb and S Roseman, J Biol Chem 235, 2529-2537 (1990)] and may be obtained, for example, either from a naturally occurring NANA aldolase-producing strain of *E. coli* (including constitutive mutant strains) or from an overexpressing recombinant strain of *E. coli*. Preferably the NANA aldolase is obtained from an over-expressing recombinant strain of *E. coli*. In
10 particular the NANA aldolase is obtained from either of the recombinant *E. coli* strains TG1[pMexAld] and TG1[pPLAld], described hereinafter and forming further aspects of the present invention.

- The NANA aldolase may be used in free form or, preferably, immobilised to allow re-
15 use of the enzyme. Immobilisation may be carried out for example by mixing untreated cell homogenate or partially purified extracts of a NANA aldolase-producing strain in a suitable buffer with an activated resin such as Eupergit C (Rohm Pharma) and either stirring or standing with occasional mixing for 2 to 15 days. The immobilised beads can then be recovered after use in the
20 reaction by filtration and re-used. Other immobilisation procedures known in the art can also be utilised, including immobilisation of the enzyme by containment within a hollow fibre or membrane reactor.

- The conversion of NAM to NANA is carried out in aqueous medium at a pH of 6.0 to
25 9.0, for example 7 to 8, at a temperature of 5° to 60°C, for example about 20° to 30°C, for from about 2 to 48 hours. The reaction is terminated when a substantial amount of NAM, up to about 95%, is converted to NANA.

- Use of NAM-enriched NAM/NAG mixtures allowing for higher concentrations of NAM
30 offers a number of advantages for NANA production, including a faster reaction, increased productivity and a lower requirement of pyruvate. Most significantly, this results in a high concentration of NANA and low concentrations of NAG and pyruvate which greatly simplifies the isolation process.

- 35 When an enriched NAM/NAG mixture is used at high concentration the pyruvate:

NAM starting molar ratio should be about 1.5 to 2.5:1. If an equilibrium mixture is employed with a lower NAM concentration a higher molar excess of pyruvate, e.g. about 5 times, is required.

5 In the third step of the process of this invention, the NANA may be isolated from the reaction mixture by any conventional physical, chemical or physiochemical method, for example by crystallisation, chromatographic separation and the like. The chosen method of isolation will be determined primarily by the concentration of NANA in the final reaction mixture and by the ratio of NANA to pyruvate and NAG. Where an
10 enriched NAM/NAG mixture has been used at high concentration with a pyruvate: NAM starting molar ratio of about 1.5 - 2.5:1 and NANA concentration in the reaction mixture is (or can be concentrated to) at least 150g/litre then simple crystallisation from the reaction mixture, e.g. by addition of from 4 to 8, preferably 6, volumes of acetic acid, is effective.

15

Where an epimerised mixture has been used without enrichment so that a high molar excess of pyruvate has been employed, or when the NANA concentration in the reaction mixture is below 150g/litre, it may be necessary to remove other components from the reaction mixture before NANA is itself isolated.

20

Sodium ions may conveniently be removed by passing the reaction mixture through an ion exchange resin such as Amberlite 200 (H⁺) or IR120 (H⁺). This reduces the pH of the reaction mixture to about 2.

25 Pyruvate may be selectively removed from the mixture after reaction with metabisulphite anion by absorption of the hydroxysulphonic acid formed onto an ion exchange resin. The reaction and adsorption may be conveniently carried out in one stage by passage of the acidified mixture through a column of quaternary ammonium ion exchange resin such as Duolite A113 in the bisulphite form. Any
30 leached bisulphite ions in the effluent from this column may be precipitated by addition of calcium ions.

NAG may be separated from NANA by retention of NANA on a suitable ion exchange resin e.g. Duolite A113 in the acetate form. NAG passes through the
35 resin and may be recycled through the process after equilibration to a NAG/NAM

mixture as above. The NANA may be eluted from the column with a suitable acid or salt such as formic acid or preferably sodium acetate. NANA may be crystallised from the effluent after suitable concentration by addition of a co-solvent such as acetonitrile or preferably acetic acid. In the case of salt elution of NANA from the ion exchange column it may be preferable to remove cations before any concentration and crystallisation by treatment of the solution with an ion exchange resin in the acid form e.g. Amberlite 200 (H+) or IR120 (H+) as above.

Crystalline NANA produced by these processes may be further treated in a number of ways. The level of solvent in the product may be reduced by drying at an elevated temperature e.g. 30° to 60° in vacuo. Alternatively, the crystalline material may be slurried in acetone containing a small amount of water (eg about 0.5%) and the crystals recovered by filtration and dried as before. The needle crystals produced by the acetic acid crystallisation may be converted either with or without prior desolvation to rhomboid crystals by adding slowly to water (eg about 1 weight per volume). After stirring for an appropriate period (eg about 30min) the crystallisation can be enhanced by the addition of acetone (eg about 8 to 9 volumes per weight initial crystals). The rhomboid crystals can be recovered by filtration, washed with acetone and dried as before. If required the rhomboid crystals can be recrystallised by repeating this procedure. NANA can thus be isolated in highly pure form.

The preparation of overexpressing recombinant E. coli NANA aldolase producing strains is described in Example 1 hereinafter.

In the Figures :-

Figure 1 shows Maps of Plasmids

- a) pMtl21 P contains a multiple cloning site within the lacZ' gene for which only the NcoI and SalI sites are indicated).
- b) pPLRES contains the bacteriophage lambda phage leftward promoter (PL) and the bacteriophage lambda phage C1857 repressor gene. The gene product from C1857 is a temperature sensitive repressor of the PL promoter.

Transcription from PL is induced by a shift from the permissive temperature to the non permissive temperature (usually 30°C to 40-42°C).

- 5 c) pMex8 contains the hybrid tac promoter which can be repressed by the product of a host cell encoded lacI gene. Repression is removed by the presence of IPTG. This plasmid also contains the rrnB transcription terminator (tt) adjacent to the tac promoter. In addition to the ColE1 origin of replication this plasmid contains the F1-intergenic region (f1).
- 10 AMP, β -lactamase gene; TET, tetracycline resistance gene; ori, origin of replication; B, BamHI; E, EcoRI; H, HindIII; N, NcoI; S, Sall. Maps are not drawn to scale.

Figure 2 shows the sequence of Plasmid pPLRES.

- 15 The sequence of one strand of plasmid pPLRES is shown, starting at the first A of the unique EcoRI site (GAATTC) and proceeding in a rightward direction (5' to 3') around the plasmid as represented in Figure 1b. The circular plasmid is written as a linear sequence.

- 20 Figure 3 shows maps of Plasmids containing the Aldolase sequence

a) pMtlAld.

b) pMexAld

25

c) pPLAJd

- 30 AMP, β -lactamase gene; TET, tetracycline resistance gene; ori, origin of replication f1, f1-intergenic region; tt, rrnB transcription terminator; triangle, promoter used to direct transcription of the aldolase sequence; tac, hybrid tac promoter; PL, bacteriophage lambda phage leftward promoter; CI857, bacteriophage lambda phage temperature sensitive repressor gene; B, BamHI; E, EcoRI; H, Hind III; N, NcoI; S, Sall. Maps are not drawn to scale.

- 35 Figure 4 shows the sequence of the cloned Aldolase fragment

The sequence includes the NcoI site (CCATGG) and the Sall site (GTCGAC) flanking the aldolase encoding sequence. The coding strand only is shown. The translation initiation codon (ATG) is located at bases 3 to 5.

5

Figure 5 shows Aldolase sequencing primers

Six synthetic DNA primers were synthesised, based on the published aldolase sequence (Y Ohta et al, Nucleic Acids Research 1985, 13, 8843-8852) and used to
10 sequence regions of the cloned aldolase gene.

The invention is illustrated by the following examples which are not intended as a limitation thereof. All temperatures are in °C.

15 **Example 1 E. ColiTG1[pMexAld] and E. ColiTG1[pPLAld]**

1. Materials

X-Gal (5-bromo-4-chloro-3-indolyl- β -D- galactopyranoside) and enzymes
20 NcoI, Sall, T4DNA ligase were obtained from Boehringer Mannheim UK (Diagnostics and Biochemicals). Taq polymerase was obtained from Promega Corporation UK. GeneClean was obtained from Bio 101, Inc. La Jolla California USA. IPTG (isopropyl- β -D-thiogalactopyranoside) was obtained from United States Biochemical. dATP, dCTP dGTP and dTTP,
25 M13mp18 and M13mp19 were obtained from Pharmacia. [α^{33} P] dATP and [α^{35} S] dATP were obtained from Amersham International PLC.

2. Plasmids and E.coli Strains

30 **a) pMtd21 P**

Obtained from Dr NP Minton [ref Chambers SP, Prior SE, Barstow DA, Minton NP, 1988 Gene 68 139-149].

b) p MexB

35 Obtained from MEDAC. Gesellschaft fur Klinische Spezialpraparate mbH

Fehlandtstrasse 3. D-2000 Hamburg 36.

c) **pPLRES**

pPLRES was constructed with the sequence shown in Figure 2.

d) **E.coli TG1**

Obtained from Amersham International plc. Geneotype: K12 Δ (lac-pro), supE, thi, hsd D5, F' tra D36, proA⁺B⁺, lacI^q, lacZ Δ M15.

e) **E.coli C600**

F⁻e14 (mcrA⁻) hr-1, leuB2, thi-1, lacY, supE₄₄, rfbD1, fhuA21.

Growth of Strains

| | | | |
|----|---------------|---------|------|
| | C600 | LB | 37°C |
| 15 | TG1 | MGT | 37°C |
| | TG1/pMex8 | MGT amp | 37°C |
| | TG1/pMexAld | MGT amp | 37°C |
| | TG1/pPLcddPCR | LBtet | 30°C |
| | TG1/pPLAld | LBtet | 30°C |
| 20 | TG1pMt21P | LB amp | 37°C |

3. **Media**

LB (per litre)

| | | |
|----|---------------|-----|
| 25 | NaCl | 10g |
| | Yeast extract | 5g |
| | Bactotryptone | 10g |
| | pH 7.0 | |

Minimal Salts (per litre)

K₂HPO₄ 7g
KH₂PO₄ 3g
5 MgSO₄·7H₂O 0.25g
(NH₄)₂SO₄ 1g
Tri-sodium citrate 0.5g
pH 7.0

MGT

Minimal salts supplemented with:

0.4% w/v glucose

0.002mg/ml thiamine HCl

MGTC

MGT supplemented with 100ml/litre of 20% w/v casamino acids solution

LB and MGT agar plates contained media supplemented with 1.5% w/v agar

20 Antibiotic supplements were at final concentration of:

100µg/ml ampicillin (amp)

5µg/ml tetracycline (tet)

4. Methods

- 25 a) DNA manipulation including ligations and transformation of E. coli strains were performed essentially as described by Maniatis et al. Molecular cloning, Cold Spring Harbour New York 1982. Transformation of pPLRES derived ligations were carried out by the standard procedure except that heat shock was for 5 minutes at 34°C and the incubation of heat shocked cells in non-selective media was at 30°C rather than at 37°C.
- 30

b) Restriction endonuclease digestions were performed using conditions recommended by the suppliers.

5 c) The nucleotide sequence of the cloned aldolase gene was determined by the M13 dideoxy sequencing method using a Sequenase kit and [α - 32 P]dATP or [α - 32 S]dATP.

10 d) Purification of DNA Fragments

Fragments of DNA were purified after electrophoresis through agarose gel by means of GeneClean glass milk as described by the supplier.

15 e) Preparation of Synthetic DNA Sequences

Synthetic DNA was prepared by means of Biosearch model SAMI (trade mark) DNA synthesiser using b-cyanoethyl phosphoramidites (ND Sinha et al, Nucleic Acid Res., 1984 12, 4539).

20 f) Amplification Reaction

25 The amplification reaction contained 1 mg genomic DNA, 1 unit Taq polymerase, 1 x Promega Taq buffer 50pmol each DNA primer, 0.0125mM each of dATP, dCTP, dGTP and dTTP in a 0.1ml final volume. The reaction was carried out in a Techne PHC-3 Thermal cycler. 1 cycle of 90°C, 10 minutes, 50°C 20 minutes then 3 cycles of 50°C, 1 minute, 70°C, 2 minutes, 92°C, 1 minute then 27 cycles of 50°C, 1 minute, 70°C, 2 minutes, 92°C, 10 seconds then 1 cycle of 50°C, 1 minute, 70°C, 5 minutes, then cooled to 4°C.

30 5. Cloning of Aldolase Encoding Sequence from *E.coli* C600

a) Genomic DNA was prepared from 5ml of an overnight culture of *E.coli* C600, grown in LB at 37°C, by the method described in Current Protocols in Molecular Biology, Vol1 page 2.4.1 (Eds FM Ausubel et al).

- b) The aldolase gene was specifically amplified from the *E.coli* C800 genomic DNA using a Taq polymerase reaction primed by synthetic DNA sequences Ald/PCR/1 and Ald/PCR/2: the design of these primers was based on the published aldolase gene sequence (Y.Ohta *et al*, Nucleic Acids Research, 1985, 13, 8843-8852).

Ald/PCR/1

5'-GACGCTACCATGGCAACGAATTTACGTGGCG-3

NcoI

Ald/PCR/2

5'-GATCCAGTCGACTCACCCGCGCTCTTGCATC-3

Sall

The DNA fragment produced by this reaction contained the aldolase encoding sequence flanked by NcoI and Sall sites introduced from the sequence in the primer. The NcoI site (CCATGG) was placed at the position corresponding to the translation initiation codon. The Sall site (GTCGAC) was placed immediately 3' to the termination codon (TGA).

- c) The aldolase gene fragment was digested with NcoI and Sall and purified by means of GeneClean.

The purified aldolase fragment was ligated into plasmid pMt121P digested with NcoI and Sall. The ligation reaction was used to transform *E.coli* TG1 to ampicillin resistance. An ampicillin resistant colony with a white phenotype on LB agar plates supplemented with ampicillin (0.1mg/ml), Xgal (0.08mg/ml) and IPTG (0.2mM) was isolated that contained the aldolase sequence. The plasmid in this clone was called pMtAld (Figure 3a).

- d) The aldolase fragment from pMtAld was transferred into M13mp18 and M13mp19 as a BamHI-HindIII fragment and the sequence determined and confirmed to be aldolase by comparison with the sequence published by Y

Ohta *et al* Nucleic Acids Res. 1985 13, 8843. The sequence of the cloned aldolase fragment in pMtIAld is shown in Figure 4. Primers used to determine the sequence were those shown in Figure 5 and the -40 primer provided with the Sequenase kit.

5

6. Cloning of Aldolase Gene into *E.coli* Expression Plasmids

10

a) Plasmid pMtIAld was digested with NcoI and Sall and the aldolase fragment purified by GeneClean.

b) Plasmid pMex8 was digested with NcoI and Sall and the 3.6 kilobase pair (Kbp) fragment purified by GeneClean.

15

c) Plasmid pPLRES was digested with NcoI and Sall and the 3.96kbp fragment purified by GeneClean.

20

d) The aldolase fragment from a) was ligated into the purified pMex8 fragment from b) and the ligation reaction used to transform *E.coli* TG1 to ampicillin resistance. A clone was isolated that contained the aldolase gene correctly inserted in the pMex8 fragment. The plasmid in this clone was called pMexAld (Figure 3b).

25

e) The aldolase fragment from a) was ligated into the purified pPLRES fragment from c) and the ligation reaction used to transform *E.coli* TGI to tetracycline resistance. A clone was isolated that contained the aldolase gene correctly inserted into the pPLRES fragment. The plasmid in this clone was called pPLAld (Figure 3c).

30

Example 2 - Production of NANA aldolase

0.5ml of a frozen suspension of *E.coli* TG1 [pMexA d] was used to inoculate 2 x 400ml of MGTA broth in 2 litre flasks.

MGTA

| INGREDIENT | g/L |
|---|-----|
| K ₂ HPO ₄ | 7 |
| KH ₂ PO ₄ | 3 |
| MgSO ₄ ·7H ₂ O | 0.1 |
| (NH ₄) ₂ SO ₄ | 1 |
| Tri-sodium citrate | 0.5 |

Distilled water. Natural pH. Sterilise for 121°/15 mins.

5 Added post-autoclaving:

| | |
|---------------|-------|
| *Thiamine.HCl | 0.002 |
| Glucose | 4 |
| *Ampicillin | 0.05 |

*filter sterilised.

10 The ampicillin solution was prepared fresh and added immediately prior to inoculation.

The flasks were incubated overnight at 37°C on a rotary shaker operated at 250 rpm with a 5mm diameter orbital motion. Each flask was used to inoculate 45 litres of MGTA/1 or MGTA/2 broth in a 70 litre fermenter.

| INGREDIENT | MGTA/1 | | MGTC/2 | |
|---|--------|-------|--------|-------|
| | g/L | g/45L | g/L | g/45L |
| K ₂ HPO ₄ | 10.5 | 472.5 | 14.0 | 630 |
| KH ₂ PO ₄ | 4.5 | 202.5 | 6.0 | 270 |
| MgSO ₄ ·7H ₂ O | 0.15 | 6.75 | 0.2 | 9 |
| (NH ₄) ₂ SO ₄ | 1.5 | 67.5 | 2.0 | 90 |
| Tri-sodium citrate | 0.75 | 33.75 | 1.0 | 45 |
| Casamino acids (Difco) | 20.0 | 900 | 20.0 | 900 |
| PPG 200 (KKGreef) | | 25 | | 25 |

Distilled water. Natural pH. Fermenters were sterilised at 121°C/30 mins.

Added post-autoclaving:

5

| | | | | |
|----------------|-------|------|-------|------|
| Glucose | 6 | 270 | - | - |
| Glycerol | - | - | 8 | 360 |
| *Thiamine. HCl | 0.002 | 0.09 | 0.002 | 0.09 |
| *Ampicillin | 0.05 | 2.25 | 0.05 | 2.25 |

*Filter sterilised. The ampicillin solution was prepared fresh and added immediately prior to inoculation.

Added two hours after inoculating:-

| | | | | |
|--|---------|------|---------|------|
| Isopropyl β -D-thio-galactoside (IPTG) | 0.024 | 1.07 | 0.024 | 1.07 |
| | (0.1mM) | | (0.1mM) | |

10 On demand:

PPG200 (KKGreef)

The cultures were incubated for 26 hours at 37°C, with stirring at 500 rpm and 45lpm airflow. IPTG was added to induce aldolase production 2 hours after inoculation.

The two fermentation broths were bulked and harvested by continuous centrifugation (Sharples) to obtain 1.75kg of wet cell paste. This was suspended in 5.5L of lysis buffer and homogenised by 3 passes through a Manton-Gaulin homogeniser (8000 psi) to obtain the crude extract.

LYSIS BUFFER

| INGREDIENT | CONCENTRATION |
|--|---------------|
| KH ₂ PO ₄ | 20mM |
| K ₂ HPO ₄ | 20mM |
| ethylenediamine tetra acetic acid (EDTA) | 1mM |
| dithiothreitol (DTT) | 1mM |
| phenylmethanesulphonylfluoride | 1mM |

The aldolase enzyme was immobilised by mixing the crude extract with 1.27kg of Eupergit-C beads (Rohm Pharma) and allowing the mixture to stand at room temperature with occasional stirring for 10 days. The beads (wet weight 4.44kg) were washed in buffer until the A₂₈₀ Of the supernatant was below 0.1, and stored in the same, but without NaCl, at 4°C.

WASH BUFFER (pH 7.0)

| INGREDIENT | CONCENTRATION |
|-----------------------------------|---------------|
| Tris-HCl | 100mM |
| NaCl | 500mM |
| EDTA | 1mM |
| DTT | 1mM |
| p-hydroxybenzoic acid ethyl ester | 3mM |

Example 3 - Production of NANA

- 5 2kg N-acetyl-D-glucosamine (NAG) and 44g sodium hydroxide were dissolved in 20 litres of water and the solution allowed to stand at room temperature. After 66 hours, 1 litre of IR120 (H⁺) (Rohm and Haas) was added to reduce the pH below 7 and then the resin removed by filtration. Three batches were epimerised to give a
- 10 bulk solution containing N-acetyl-D-mannosamine (NAM) and NAG in the approximate ratio of 1:4.
- 50 litres of the epimerised mixture and 2.5kg of sodium pyruvate were added to a 70 litre vessel. The pH was adjusted to 7.4 with sodium hydroxide and the reaction
- 15 started by the addition of 4.4kg of washed immobilised enzyme beads from Example 2. The reaction mixture was stirred at 133 rpm with a single turbine impeller, and the temperature maintained at 20°C. The reaction was allowed to proceed for 27 hours after which approximately 90% of the NAM had been converted to NANA.
- 20 The immobilised enzyme beads were removed from the reaction mixture by vacuum filtration through filter cloth on a pan filter, washed with 7L distilled water and the washes bulked with the filtrate. Sodium ions were removed from the filtrate by passage through an IR120.(H⁺) resin column (15L), at a flow rate of 2 bed volumes (bv) hr.⁻¹. NAG, NAM, NANA and pyruvate passed straight through the column.
- 25 Residual NAG, NAM, NANA and pyruvate were washed from the column with 1 bv of distilled water, at the same flow rate, and bulked with the column effluent.

Pyruvate was absorbed from the effluent solution onto a Duolite A113 PLUS (HSO_3^-) (Rohm and Haas) column (16.7L) for column regeneration method see below). NAG, NAM and NANA passed through the column; residual amounts were removed by washing with 1 bv of distilled water and bulked with the column effluent. Sodium ions remaining in solution were removed by treating the effluent with 2.2kg (wet weight) of IR120.(H^+) resin. The resin was removed by vacuum filtration through Whatman 54 filter paper and washed with 2L of distilled water, the washes were bulked with the filtrate. Excess HSO_3^- was precipitated by the addition of 800g of solid calcium hydroxide, the solid being recovered by filtration as before. Calcium ions were removed by the batchwise addition of 3kg (wet weight) of IR120.(H^+) resin, the resin being recovered by filtration as before.

NANA was adsorbed from the filtrate onto a Duolite A113 PLUS.(OAc^-) column (25L). NAG and NAM passed straight through the column and were washed clear with distilled water. NANA was eluted from the column with 1M-sodium acetate solution. The fraction eluting between 0.5-2 bv was bulked and excess Na^+ was removed on a column of IR120.(H^+) resin (37.5L). The NANA was washed from the column with 0.5 bv distilled water and bulked with the column effluent.

The effluent was concentrated to approximately 100g.L^{-1} NANA on a Balfors wiped film evaporator, vacuum filtered through Whatman 54 filter paper to remove insoluble material and the NANA crystallised by the addition of 9 volumes of acetonitrile. The solid was recovered by filtration as before and washed with fresh acetonitrile, before drying to constant weight over P_2O_5 in a vacuum oven. This solid (838g) was approximately 80% pure by HPLC.

The above solid (818g) was recrystallised by redissolving in water (at 250g.L^{-1}) and adding 5 volumes of glacial acetic acid. The solid was recovered after 3 days at 4°C by vacuum filtration through Whatman 54 filter paper and washed with 5L of acetonitrile. The solid was dried under vacuum at a temperature of 80°C to constant weight. Final weight 620.4g, purity by HPLC 95.4%.

Regeneration of A113 PLUS.HSO₃⁻ Resin

The resin was supplied in the Cl⁻ form. The following solutions were then passed through the column.

1. New Resin

- Stage
- i) 0.5M-Sodium hydroxide to give an effluent pH>12
 - ii) Distilled water to neutral pH
 - iii) 0.5M-HCl to give an effluent pH<2
 - iv) Distilled water to neutral pH
 - v) 0.5M sodium metabisulphite solution (2 bv)
 - vi) Distilled water to remove excess Na₂S₂O₅

2. Used Resin

For used resin only stages v) and vi) were required.

Example 4 - Production of NANA

(I) Preparation of NAM-enriched Mixtures

A solution of NAG (1.75kg) and sodium hydroxide (38g) in water (4.37L) was incubated at 25° for 40 hr. Amberlite 200 (H⁺) resin (800ml) was added to reduce the pH below 7 and the resin removed by filtration. 2 x 1L aliquots of the resulting filtrate were treated as follows:

- a) A 1L portion was evaporated to give an oily solid. This was stirred with methanol (660ml) at room temperature until a fine white solid remained. This was removed by filtration and washed with methanol (100ml) to give, after drying *in vacuo* at 60°, 194.8g of NAG. The filtrate was evaporated to give an oily solid (91.1g after drying) which was dissolved in water (180ml). This solution was assayed by ion-exclusion HPLC (cf. Kragl, Gygas, Ghisalba and Wandrey in Angew. Chem. Int. Ed. Eng. Vol.30, 1991, p827-828) and found to contain 205g/L NAM, 85g/L NAG (NAM:NAG 2.5:1).

b) The second 1L portion was mixed with isopropanol (5L) and left at room temperature for 3 days. The resulting crystals were filtered off (paper), washed with isopropanol (200ml) and dried *in vacuo* at 60° to give NAG (159g). The mother liquors were evaporated to an oily solid which was extracted with methanol (660ml) at room temperature. The residual solid was filtered off and dried *in vacuo* at 60° (56.4g). This solid was found still to contain NAM (NAG:NAM c 3:1) so was re-extracted with methanol to give, after drying, NAG (41.8g). The filtrate and wash were combined and evaporated to give an oily solid (14.8g) which was very rich in NAM. This was combined with the oily residue obtained by evaporation of the first filtrate and washes (80.3g) by dissolving in 167ml of water. Assay of this solution by ion-exclusion HPLC gave 224g/L NAM, 66g/L NAG (NAM:NAG 3.4:1).

(ii) Aldolase Reaction

To 20ml of the NAM-enriched mixture from method b) (4.48g NAM, 1.32g NAG) was added sodium pyruvate (4.54g, 2:1 molar ratio pyruvate:NAM) and enzyme beads (8g wet weight, from Example 2). The reaction mixture was incubated with stirring at 20° for 48h.

(iii) Isolation of NANA

At the end of the reaction, the reaction mixture was filtered to remove the enzyme beads and the filtrate (20ml) was passed through an Amberlite 200 (H⁺) column (40ml). NANA was displaced with 1bv of distilled water, but a further 25ml was needed to achieve good recovery. The eluate was evaporated back to 20ml. Acetic acid (5 volumes) was added and the mixture was left at 4°C for 3 days to crystallise. Needle crystals were removed by filtration and dried *in vacuo* at 60° to give 3.8g solid, assaying 95% NANA.

Example 5 - Production of NANA

Epimerisation and Preparation of NAM-enriched mixtures

5 5kg NAG and 110g sodium hydroxide were dissolved in 12.5 litres of distilled water and the solution allowed to stand at 25°C. After 65 hours 3.3 litres of Amberlite 200 (H⁺) (Rohm and Haas) was added to reduce the pH below 7.0 and the resin removed by filtration. The epimerised solution contained NAM and NAG in the approximate ratio of 1:4.

10

NAG was partially crystallised from the epimerised solution by the addition of propan-2-ol (5 volumes), the solid being recovered after 4 days at 4°C by filtration, and washed with fresh solvent. The propan-2-ol solution was concentrated approximately 6 fold on a Balfors wiped film evaporator giving a second crop of NAG
15 which was recovered by filtration as before. The filtrate was evaporated to dryness on a Buchi rotary evaporator and extracted with a total of 14 litres of methanol. A further crop of NAG was removed at this time. The methanol solution was evaporated to dryness as before, the solid obtained was dissolved to 4.17 litres in water and insoluble material removed by filtration. This solution had a NAM to NAG
20 ratio of approximately 4.5:1.

Bioconversion/Isolation

3.5 litres of the epimerised NAM-enriched mixture (620g NAM, 133g NAG) and 442g
25 of sodium pyruvate were added to a 7 litre vessel. The pH was adjusted to 7.5 with sodium hydroxide and the reaction started by the addition of 1.7kg of washed immobilised enzyme beads from Example 2. The reaction mixture was stirred at 430rpm with a single marine impeller, and the temperature maintained at 20°C. The reaction was allowed to proceed for 46 hours after which the enzyme beads
30 were removed from the reaction mixture by vacuum filtration through filter cloth and washed with 1L distilled water.

NANA was crystallised from the filtrate by the addition of 5 volumes of glacial acetic acid (22.5L). The solid was recovered after 5 days at 4°C by vacuum filtration
35 through Whatman 54 paper and washed with fresh acetic acid (4L). Excess acetic

acid was removed from the solid by washing with acetone (24L) and the solid dried to constant weight at 50°C in a vacuum oven. Final weight 576.4g, purity by HPLC 96.4%.

5 **Example 6 - Production of NANA**

(i) **Epimerisation and Preparation of NAM-enriched mixtures**

Sodium hydroxide pellets (5.0g) were dissolved in process water (1250 ml), NAG
10 (500g) added and the suspension stirred until solution was obtained. The solution was allowed to stand at 33°C for 15 hours when the NAM/NAG ratio was 0.21. The solution was neutralised with glacial acetic acid and then concentrated to 986 ml. Six volumes of isopropanol was added and the suspension stirred for 6 hours. Crystallised NAG was filtered off, washed with isopropanol (300 ml) and dried to
15 give 342g NAG.

(ii) **Bioconversion**

The combined filtrate and wash (6.30L) was concentrated to 950 ml, and additional
20 process water added to chase off residual isopropanol. The final concentrate (935 ml) was treated with 86.8g sodium pyruvate and the pH adjusted to 7.4 with glacial acetic acid. NANA aldolase (40% w/v) was added and the suspension stirred for 25½ hours at 25°.

25 (iii) **Isolation**

The enzyme was filtered off and washed with 2 x 750 ml process water. The filtrate and washes (1.74L) were concentrated to 395 ml, to which was added with swirling
30 2370 ml of glacial acetic acid and a small quantity of seed. The crystallised suspension was allowed to stand for 7.5 hours at 21°C and then filtered. The cake was washed with 118 ml of 85% acetic acid 15% water followed by isopropanol (590 ml) and dried in vacuo at 50°C. The dried solid (90.9g) assayed at 96.8%.

Example 7 - Production of NANA aldolase

E. coli TG1[pMexAld] from a freeze dried ampoule was rehydrated using nutrient broth (Oxoid), streaked onto the surface of MGTA agar and incubated at 37°C overnight. Bacteria from areas of the plate having separate colonies were suspended in Brain Heart Infusion broth (Oxoid) containing 10% glycerol and stored in 0.5 ml amounts at -20°C for future use.

A 2 litre flask containing 400 ml of MGTA medium (see Example 2) was inoculated with 0.5 ml of stored (-20°C) suspension and incubated overnight at 37°C on an orbital shaker (50 mm throw) rotating at 250 rpm. The contents of the flask were used to inoculate a fermenter containing 4.5 L of MGTA. The culture in the fermenter was incubated at 37°C with aeration at 4.5 L/min and stirring at 500 rpm until a sharp fall in exhaust CO₂ concentration indicated the end of logarithmic growth (4.5 - 6 hours). The contents of the fermenter were used to inoculate a larger vessel containing 450L of LPSG1 medium maintained at 37°C with stirring at 350 rpm. The air flow to the vessel was set at 450 L/min and when the exhaust CO₂ concentration reached 2% (5 - 5.5 hr) a solution containing 10.7 g of IPTG (isopropyl β -D-thiogalactoside) was pumped into the fermenter. The culture was harvested 18 hours after inoculation.

LPSG1 Medium

| Ingredients | g/L |
|--------------------------|-----|
| Lab Lemco Powder (Oxoid) | 40 |
| Peptone L37 (Oxoid) | 40 |
| NaCl | 5 |
| Glycol | 50 |

The cell paste was further treated as described in Example 2.

Example 8 - Production of NANA aldolase

- E. coli* TG1 [pP_LIdl from a freeze dried ampoule was rehydrated using nutrient broth (Oxoid), streaked onto the surface of nutrient agar (Oxoid) containing 5 mg/L of tetracycline hydrochloride and incubated at 30°C overnight. Bacteria from areas of the plate having separate colonies were suspended in Brain Heart Infusion broth (Oxoid) containing 10% glycerol and stored in 1 ml amounts at -20°C for future use.
- 10 A 2 litre flask containing 400 ml of MCGTT medium was inoculated with 0.8 ml of stored (-20°C) suspension and incubated overnight at 30°C on an orbital shaker (50 mm throw) rotating at 250 rpm. The contents of the flask were used to inoculate a fermenter containing 450 L of LPSG1 (see Example 7) medium maintained at 30°C with stirring at 200 rpm and an air flow of 450 L/min. After 9 hours, the fermentation
- 15 temperature was raised to 42°C to induce aldolase production. The culture was harvested 24 hours after inoculation.

MCGTT Medium

| Ingredients | g/L |
|---|-----|
| K ₂ HPO ₄ | 7 |
| K ₂ HPO ₄ | 3 |
| MgSO ₄ ·7H ₂ O | 0.1 |
| (NH ₄) ₂ SO ₄ | 1 |
| Tri-sodium citrate | 0.5 |
| Casamino Acids (Difco) | 20 |

20

Dissolve in distilled water. Natural pH. Sterilise at 121°C for 15 min.

Added post-autoclaving

| Solution | Conc. (g/L) | Sterilisation | Volume (ml/L) | Final conc. (g/L) |
|------------------|-------------|---------------|---------------|-------------------|
| Glucose | 400 | 121°C 15 min | 10 | 4 |
| Thiamine.HCL | 1 | Filtration | 2 | 0.002 |
| Tetracycline.HCL | 2.5 | Filtration | 2 | 0.005 |

- 5 Freshly-prepared tetracycline solutions were added to the medium immediately before inoculation.

The cell paste was further treated as described in Example 2.

10 **Example 9 - Production of NANA**

(i) **Epimerisation**

- 15 Sodium hydroxide pellets (1.96g) were dissolved in water (490ml), NAG (196g 7th cycle of use) was added and the suspension stirred until solution was obtained. The solution was allowed to stand at 33°C for 19 hours when the NAM/NAG ratio was 0.21. The solution was neutralised with glacial acetic acid.

(ii) **NAM/NAG Enrichment**

- 20 The solution was concentrated to 380 mls (0.63 of its original volume). Six volumes of isopropanol (2280 ml) were added and the suspension stirred for 5.5 hours (NAM/NAG ratio 2.14). Crystallised NAG was filtered off, and washed with isopropanol (196ml) and dried in a vacuum oven at 50°C overnight to give NAG
25 (130.1g).

(iii) Bioconversion

The combined filtrate and wash (2660 ml) from NAG filtration was concentrated to 345 ml, and additional process water added to chase off residual isopropanol. The final concentrate (300ml NAM concentration 90.5 g/l) was treated with sodium pyruvate (27.12g) (2.15 M sodium pyruvate : 1 M NAM) and the pH adjusted to 7.4 with glacial acetic acid. NANA aldolase was added and the suspension stirred for 24 hours at 25°C, this resulting in a conversion of 90%.

(iv) Isolation

The enzyme was filtered off and washed with water (240ml). The filtrate and washes (590ml) were concentrated to 138 ml, to which was added with mixing 816 ml (6 vols) of glacial acetic acid and a small quantity of seed. The crystallised suspension was allowed to stand for 17 hours at 21°C and then filtered. The cake was washed with 41 ml (0.3 vol of 85% acetic acid 15% water followed by acetone (204 mls - 1.5 vol). The wet cake (62.93g) was then used for subsequent work.

(v) Acetone Desolvation

Wet cake (30g) was added to 0.5% aqueous acetone (268ml) and the suspension was stirred at room temperature (21°C) for 2 hours, and then filtered. The cake was washed with acetone (2 x 40 mls), and dried in a vacuum oven at 50°C overnight to give desolvated NANA (12.44g) (Potency 99.2%, corrected for water content).

(vi) Rhomboid Crystallisation

Wet cake (30g) was added slowly to water (27 mls) whilst stirring. The suspension was stirred for 30 minutes at 21°C, by which time the wet cake had converted to rhomboids. Acetone (240 mls) was added over 30 minutes. After acetone addition the mixture was stirred for 1.5 hours at 21°C. The mixture was then cooled to 5°C for 1 hour and filtered. The cake was washed with acetone (2 x 40mls), and dried in a vacuum oven at 35°C overnight to give NANA rhomboids (13.44g). (Potency 99.4%, corrected for water content).

Example 10 - Crystallisation of Rhomboid Crystals

5 N-Acetyl-D-Neuraminic rhomboid crystals (56g) were added to stirred water (100ml) at room temperature over 25 - 30 minutes. Initially solution occurred but rhomboid crystals later precipitated. The slurry was stirred for 30 minutes and acetone (800mls) added over 30 minutes. The slurry was stirred for 2 hours at room temperature. The solids were filtered, washed with a acetone water mixture (8:1, 100ml) followed by acetone (300ml) and dried in vacuo at 35°C overnight. Yield
10 solid, 94.1% w/w.

Claims

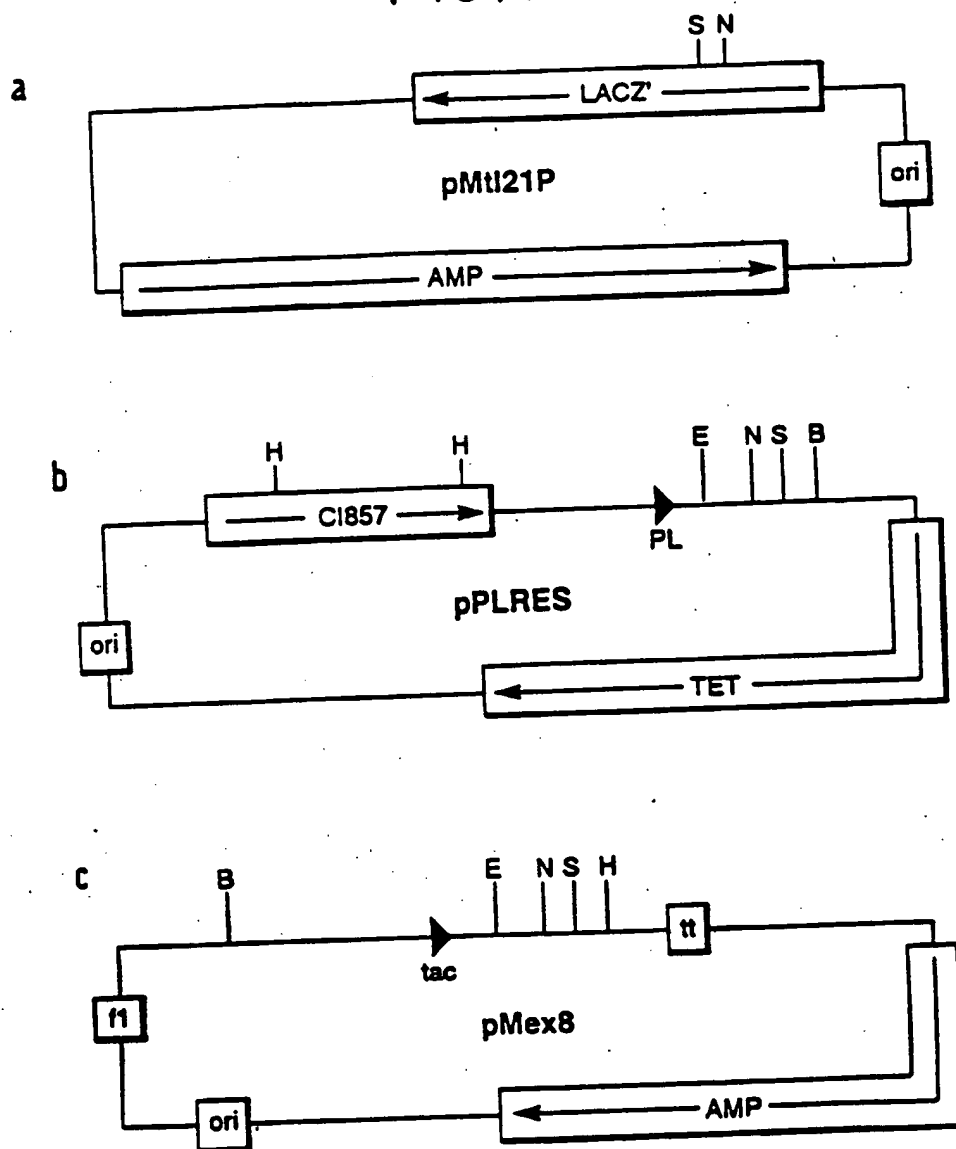
1. A process for the preparation of N-acetyl-D-neuraminic acid (NANA), which process comprises the steps of: -
5
 1. converting N-acetyl-D-glucosamine (NAG) to N-acetyl-D-mannosamine (NAM) by base-catalysed epimerisation;
 2. reacting NAM with pyruvate under enzymatic conditions; and
 3. isolating the thus - produced NANA.
- 10 2. A process as claimed in Claim 1 wherein epimerisation is carried out at the saturation concentration of NAG.
- 15 3. A process as claimed in Claim 1 or Claim 2 wherein the reaction mixture is neutralised prior to step 2.
4. A process as claimed in any one of claims 1 to 3 wherein the NAM/NAG mixture obtained by step 1 is enriched in NAM by selective removal of NAG.
- 20 5. A process as claimed in Claim 4 wherein enrichment is effected by treatment of the NAM/NAG mixture with an organic solvent and removal of crystalline NAG.
- 25 6. A process as claimed in Claim 5 wherein the organic solvent is a lower alcohol.
7. A process as claimed in claim 5 or claim 6 wherein the organic solvent is isopropanol.
- 30 8. A process as claimed in Claim 7 wherein the ratio of isopropanol: NAM/NAG mixture is 5:1 to 10:1.
9. A process as claimed in claim 7 or Claim 8 wherein further enrichment is effected by evaporation of the isopropanol/water azeotrope.

10. A process as claimed in any one of claims 5 to 9 wherein the enrichment is effected at a temperature of from 15° to 30°C
- 5 11. A process as claimed in any one of claims 4 to 10 wherein the pyruvate : NAM starting molar ratio for step 2 is about 1.5:1 to 2.5:1.
12. A process as claimed in claim 11 wherein NANA is isolated by crystallisation from the reaction mixture.
- 10 13. A process as claimed in claim 12 wherein crystallisation of NANA from the reaction mixture is effected by addition of from 4 to 8 volumes of acetic acid.
14. A process as claimed in any one of claims 4 to 13 wherein the NAG removed from the NAM/NAG mixture is reused in step 1 of the process.
- 15 15. A process as claimed in any one of claims 1 to 14 wherein the enzymatic conditions comprise incubation in the presence of NANA aldolase.
16. A process as claimed in claim 15 wherein the NANA aldolase is obtained from an over-expressing recombinant strain of E.coli.
- 20 17. A process as claimed in claim 16 wherein the recombinant strain of E.coli is TG1[pMexAld] or TG1[pPLAld].
- 25 18. A process as claimed in any one of claims 15 to 17 wherein the NANA aldolase is immobilised.
19. A process as claimed in any one of claims 1 to 18 wherein step 2 is effected at pH 6 to 9 and temperature 5° to 60°C.
- 30 20. A process as claimed in any one of claims 1 to 19 wherein NANA is isolated as needle - shaped crystals.
21. A process as claimed in any of claims 1 to 19 wherein NANA is isolated as rhombic crystals.
- 35

22. A process as claimed in any one of claims 1 to 20 which process comprises the further step of converting needle - shaped crystals of NANA to rhombic crystals of NANA .

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FIG. 1



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FIG. 2(1/5)

1 AATCTTACA CTTAGTTAAA TTGCTAACTT TATAGATTAC AAAACTTAGG
51 AGGGTTTTTA CCATGGTACC TTAAACCCCG GTCGACGGA TCCTGCAGCC
101 CAGCTTGGGG ACCCTAGAGG TCCCCTTTT TATTTGAAT TGGGAGATCC
151 CAATTCTCAT GTTGACAGC TTATCATCGA TAAGCTAGCT TTAATGCGGT
201 AGTTTATCAC AGTTAAATTG CTAACGCAGT CAGGCACCGT GTATGAAATC
251 TAACAAATGCG CTCATCGTCA TCCTCGGCAC CGTCACCCTG GATGCTGTAG
301 GCATAGGCTT GGTATGCCG GTACTGCCGG GCCTCTTGGG GGATATCGTC
351 CATTCCGACA GCATCGCCAG TCACTATGGC GTGCTGCTAG CGCTATATGC
401 GTTGATGCAA TTTCTATGCG CACCCGTTCT CGGAGCACTG TCCGACCGCT
451 TTGGCCGCCG CCCAGTCCTG CTCGGCTTCCG TACTTGGAGC CACTATCGAC
501 TACGCGATCA TGGCGACCAC ACCCGTCTG TGGATTCTCT AGGCCGGACG
551 CATCGTGGCC GGCATCACCG GCGCCACAGG TCGGGTTGCT GCGGCCCTATA
601 TCGCCGACAT CACCGATGGG GAAGATCGGG CTCGCCACTT CGGGCTCATG
651 AGCGCTTGTT TCGGCGTGGG TATGTTGGCA GCGCCCGTGG CCGGGGGACT

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701 GTTGGGCGC ATCTCCTTGC ACGCACCATT CCTTGGCGG GCGGTGCTCA
751 ACGGCTCAA CCTACTACTG GGCTGCTTCC TAATGCAGGA GTCGCATAAG
801 GGAGAGCGTC GTCCGATGCC CTTGAGAGCC TTCAACCCAG TCAGCTCCTT
851 CCGGTGGCG CCGGGCATGA CTATCGTCGC CGCACTTATG ACTGTCTTCT
901 TTATCATGCA ACTCGTAGGA CAGGTGCCG CAGCGCTCTG GGTCAATTTT
951 GCGGAGGACC GCTTTCGCTG GAGGCGGAG ATGATCGGCC TGTCGCTTGC
1001 GGATTTCGGA ATCTTGCAG CCCTCGCTCA AGCCTTCGTC ACTGGTCCCG
1051 CCACCAACG TTTCGGCGAG AAGCAGGCCA TTATCGCCGG CATGGCGGCC
1101 GACGCGCTGG GCTACGTCTT GCTGGCGTTC GCGACGCGAG GCTGGATGGC
1151 CTTCCCCATT ATGATTCTTC TGGCTTCCGG CGGCATCGGG ATGCCCGCGT
1201 TGCAGGCCAT GCTGTCCAGG CAGGTAGATG ACGACCATCA GGGACAGCTT
1251 CAAGGATCGC TCGCGGCTCT TACCAGCCTA ACTTCGATCA CTGGACCGCT
1301 GATCGTCAG GCGATTTATG CCGCCTCGGC GAGCACATGG AACGGGTTGG
1351 CATGGATTGT AGGCGCCGCC CTATACCTTG TCTGCCCTCCC CGCGTTGCGT
1401 CGCGGTGCAT GGAGCCGGGC CACCTCGACC TGAATGGAAG CCGGCGGCAC
1451 CTCGCTAACG GATTACCCAC TCCAAGAATT GGAGCCAATC AATTCTTGGC

FIG. 2
(2/5)

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FIG. 2
(3/5)

1501 GAGAACTGTG AATGCGCAAA CCAACCCTTG GCAGACATA TCCATCGCGT
1551 CCGCCATCTC CAGCAGCCGC ACGGGCGCA TCTCGGGGA TGATCAGCTG
1601 CCTCGCGCGT TTCGGTGATG ACGGTGAAA CCTCTGACAC ATGCAGCTCC
1651 CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAAGCC
1701 CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCG CAGCCATGAC
1751 CCAGTCACGT AGCGATAGCG GAGTGTATAC TGGCTTAACT ATCGGGCATC
1801 AGAGCAGATT GTACTGAGAG TGCACCATAT GCGGTGTGAA ATACCGCACA
1851 GATGCGTAAG GAGAAATAC CGCATCAGGC GCTCTTCCGC TTCCTCGCTC
1901 ACTGACTCGC TCGGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA
1951 CTCAAAGGCG GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA
2001 AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAGGCC
2051 GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG AGCATCACAA
2101 AATCGACGC TCAAGTCAGA GGTGGCGNAA CCCGACAGGA CTATAAAGAT
2151 ACCAGGGGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCGGACC
2201 CTGCCGCTTA CCGGATACCT GTCCGCCCTT CTCCCTTCGG GAAGCGTGGC
2251 GCTTCTCTCA TGCTCAGCT GTAGGTATCT CAGTTCGGTG TAGGTGCTTC
2301 GCTCCAAGCT GGGCTGTGTG CAGGAACCC CCGTTCAGCC CGACCGCTGC

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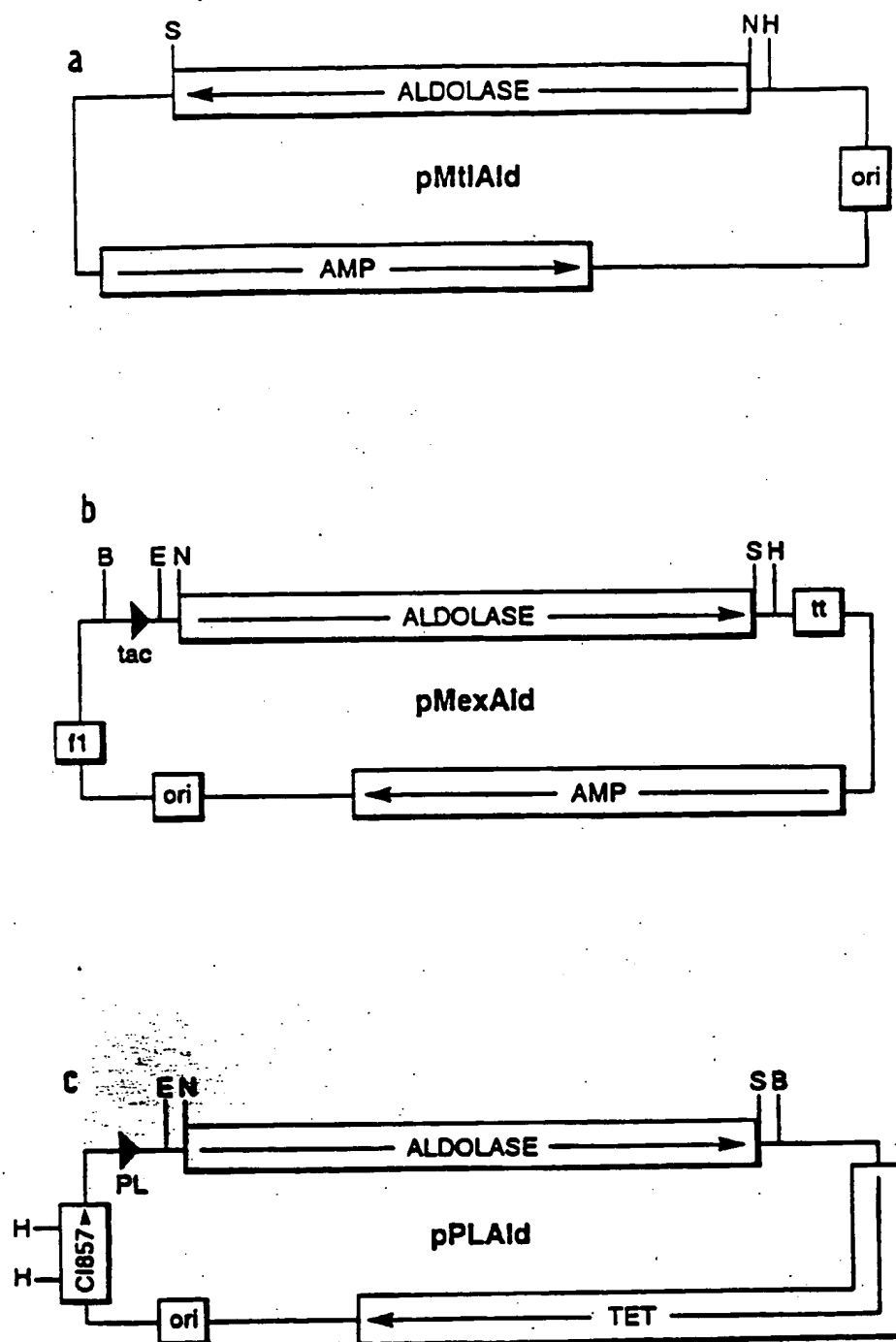
2351 GCCTTATCCG GTAACATATCG TCTTGAGTCC AACCCGGTAA GACACGACTT
2401 ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG
2451 TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT
2501 AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG
2551 AAAAAGAGTT GGTAGCTCTT GATCCGGCNA ACAAAACCACC GCTGGTAGCG
2601 GTGGTTTTT TGTGTGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT
2651 CAAGAAGATC CTTTGATCTT TTCTACGGG TCTGACGCTC AGTGGAACGA
2701 AAATCAGCT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA
2751 CCTAGATCCT TTTTCAGATCT CCCGATCTTT AGCTGTCTTG GTTTGCCCAA
2801 AGCGCATTCG ATAATCTTTC AGGGTTATGC GTTGTTCCTT ACAACCTCCT
2851 TAGTACATGC AACCATTATC ACCGCCAGAG GTAAAAATAGT CAACACGCAC
2901 GGTGTTAGAT ATTTATCCCT TCGGGTGATA GATTTAACGT ATGAGCACAA
2951 AAAAGAAACC ATTAACACAA GAGCAGCTTG AGGACGCACG TCGCCTTAAA
3001 GCAATTTATG AAAAAAGAA AAATGAACTT GGCTTATCCC AGGAATCTGT
3051 CGCAGACAAG ATGGGGATGG GGCAGTCAGG CGTTGGTGCT TTATTTAATG
3101 GCATCAATGC ATTAATGCT TATAACGCCG CATTGCTTAC AAAAATTCCTC
3151 AAAGTTAGCG TTGAAGAATT TAGCCCTTCA ATCGCCAGAG AAATCTACGA
3201 GATGTATGAA GCGGTTAGTA TGCAGCCGTC ACTTAGAAGT GAGTATGAGT

FIG. 2
(4/5)

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3251 ACCCTGTTTT TTCTCATGTT CAGGCAGGGA TGTTCTCACC TAAGCTTAGA
3301 ACCTTTACCA AAGTGATGC GGAGAGATGG GTAAGCACAA CCAAAAAGC
3351 CAGTGATTCT GCATTCTGGC TTGAGGTTGA AGGTAATTCC ATGACCGCAC
3401 CAACAGGCTC CAAGCCAAGC TTTCTCTGACG GAATGTTAAT TCTCGTTGAC
3451 CCTGAGCAGG CTGTTGAGCC AGTGATTTC TGCATAGCCA GACTTGGGGG
3501 TGATGAGTTT ACCTTCAAGA AACTAATTAG GGATAGCGGT CAGGTGTTTT
3551 TACAACCACT AAACCCACAG TACCCAAATGA TCCCATGCCAA TGAGAGTTGT
3601 TCCGTTGTGG GGAAAGTTAT CGTAGTCAG TGGCCTGAAG AGACGTTTGG
3651 CTGATCGGCA AGGTGTTCTG GTCGGCGCAT AGCTGATAAC AATTGAGCAA
3701 GAATCTTCAT CGGGGCTGCA GCCCACGATG CGTCCGGCGT AGAGGATCTC
3751 TCACCTACCA AACAAATGCCC CCTGCAAAA AATAAATTCA TATAAAAAAC
3801 ATACAGATAA CCATCTGCGG TGATAAATTA TCTCTGGCGG TGTGACATA
3851 AATACCACTG GCGGTGATAC TGAGCACATC AGCAGGACGC ACTGACCACC
3901 ATGAAGGTGA CGCTCTTAAA ATTAAGCCCT GAAGAAGGGC AGCATTCAAA
3951 GCAGAAGGCT TTGGGGTGTG TGATACGAA CGAAG

FIG.2
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FIG. 3^{7/9}

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FIG. 4 (1/2)

1 CCATGGCAAC GAATTACGT GCGTAATGG CTGCACTCCT GACTCCTTTT
51 GACCAACAAC AAGCACTGGA TAAAGCGAGT CTGCGTCGCC TGGTTCAGTT
101 CAATATTGAG CAGGGCATCG ACGGTTTATA CGTGGGTGGT TCGACCCGGAG
151 AGGCCTTTGT ACAAGCCTT TCCGAGCGTG AACAGGTACT GGAAATCGTC
201 GCCGAAGAGG CGAAAGGTAA GATTAACTC ATCGCCACG TCGGTTGCGT
251 CAGCACCGCC GAAAGCCAAC AACTTGCGGC ATCGGCTAAA CGTTATGGCT
301 TCGATGCCGT CTCCGCCGTC ACGCCGTTCT ACTATCCTTT CAGCTTTGAA
351 GAACACTGCG ATCACTATCG GGCAATTATT GATTCGGCGG ATGGTTTGCC
401 GATGGTGGTG TACAACATTC CAGCCCTGAG TGGGGTAAAA CTGACCCCTGG
451 ATCAGATCAA CACACTTGTT ACATTGCCCTG GCGTAGGTGC GCTGAACACAG
501 ACCTCTGGCG ATCTCTATCA GATGGAGCAG ATCCGTCGTG AACATCCTGA
551 TCTTGTGCTC TATAACGGTT ACGACGAAAT CTTCCGCTCT GGTCTGCTGG
601 CGGGCGCTGA TGGTGGTATC GGCAGTACCT ACAACATCAT GGGCTGGCGC

9 / 9

651 TATCAGGGGA TCGTTAAGC GCTGAAAGAA GCGATATCC AGACCGCGCA
 701 GAAACTGCAA ACTGAATGCA ATAAAGTCAT TGATTACTG ATCAAAAACGG
 751 GCGTATTCCG CGGCCTGAAA ACTGTCCTCC ATTATATGGA TGTCGTTTCT
 801 GTGCCGCTGT GCCGCAACC GTTTGGACCG GTAGATGAAA ATATCTGCC
 851 AGAACTGAAG GCGCTGGCCC AGCAGTTGAT GCAAGAGCCG GGGTGAGTCG
 901 AC

FIG. 4(2/2)

SequencePrimer Number

| | |
|----------------------------|---|
| 5'-CGCCGAAGAGCGCAAAGGTA-3' | 1 |
| 5'-CCAGCCCTGAGTGGGTAA-3' | 2 |
| 5'-TGGGCTGGCGCTATCAGGGG-3' | 3 |
| 5'-GCGCGGTCTGGATATCGCCT-3' | 4 |
| 5'-AGGCAATGTAACAAGTGTGT-3' | 5 |
| 5'-GCGGTGCTGACGCAACCGAC-3' | 6 |

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 94/01860

A. CLASSIFICATION OF SUBJECT MATTER

C 12 P 19/26

According to International Patent Classification (IPC) or to both national classification and IPC 5

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C 12 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| X | EP, A1. 0 428 947 (FORSCHUNGSZENTRUM JÜLICH et al.) 29 May 1991 (29.05.91). claims 1,7. | 1, 10, 19 |
| X.P | WO, A1. 93/15 214 (MARUKIN SHOYU) 05 August 1993 (05.08.93). abstract. | 1 |

☐ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

21 September 1994

Date of mailing of the international search report

19.10.94

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ANHANG

zum Internationalen Recherchen-
bericht über die internationale
Patentanmeldung Nr.

ANNEX

to the International Search
Report to the International Patent
Application No.

ANNEXE

au rapport de recherche inter-
national relatif à la demande de brevet
international n°

PCT/EP 94/01860 SAE 91741

In diesem Anhang sind die Mitglieder
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| Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche | Datum der Veröffentlichung Publication date Date de publication | Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets | Datum der Veröffentlichung Publication date Date de publication |
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